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# Cotton benzoquinone reductase: Up-regulation during early fiber development and heterologous expression and characterization in *Pichia pastoris*☆

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## Abstract

Benzoquinone reductase (BR; EC 1.6.5.7) is an enzyme which catalyzes the bivalent redox reactions of quinones without the production of free radical intermediates. Using 2D-PAGE comparisons, two proteins were found to be up-regulated in wild-type cotton ovules during the fiber initiation stage but not in the fiberless line SL 1-7-1. These proteins were excised from the gel, partially sequenced and identified to be BR isoforms. PCR was used to amplify both full length coding regions of 609 bp and once cloned, the restriction enzyme HindIII was used to distinguish the clones encoding the BR1 (one site) and BR2 (two sites) isoforms. Both deduced protein sequences had 203 residues which differed at 14 residues. The molecular mass and p*I*s were similar between the measured protein (2D-PAGE) and the theoretical protein (deduced). Heterologous proteins BR1 and BR2 were produced for further study by ligating the BR1 and BR2 clones in frame into the α-factor secretion sequence in pPICZαA vector and expressed with *Pichia pastoris*. Both BR1 and BR2 were approximately 26.5 kDa and did enzymatically reduce 2,6-dimethoxybenzoquinone similar to the fungal BR.
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*Keywords:* Benzoquinone reductase; Fiber development; *Gossypium hirsutum*; Quinones; *Pichia pastoris*

## 1. Introduction

Benzoquinone reductase (NADH:2-hydroxy-1,4-benzoquinone oxidoreductase, BR, EC 1.6.5.7) protein family consist of a group of flavoproteins which have been reported to catalyze bivalent redox reactions which avoid the production of free radical intermediates. These reactions include the reduction of para- and orthoquinones as well as 2,6-dichlorophenolindophenol and the one-electron acceptor ferricyanide [3,12]. The enzyme BR has been well characterized in the white rot fungus, *Phanerochaete chrysosporium*, and brown rot fungus, *Gloeophyllum trabeum*, due to its possible role in the bioremediation of aromatic pollutants and in the degradation of lignin [1,3,15,21]. In these fungi, two extracellular proteins, lignin peroxidase and manganese peroxidase, catalyze the initial step in lignin degradation [1,3]. The BR is proposed to function by catalyzing the further breakdown of monomeric intermediates.

BRs have been found in the haustoria of parasitic plants and in the roots of non-parasitic plants exposed to allelopathic quinones in the rhizosphere [12]. In parasitic plants, BR likely functions in a similar manner as in fungi, in that it is involved in the further breakdown of monomeric intermediates from cell wall degradation. Interestingly, one of the substrates for BR is 2,6-dimethoxybenzoquinone (DMBQ) which has been

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identified as a common constituent of cell walls in at least 27 families of plants [8]. Additional reports indicate that DMBQ is an active haustoria inducing factor in the parasitic plant *Striga* [4] and in the parasitic species of Scrophulariaceae [17]. In Scrophulariaceae, the induction of haustoria by quinones occurs through the rhizosphere [17].

The function of BR in parasitic plant haustoria and in plant—plant interactions are becoming more established. In an earlier study, we evaluated 2D-PAGE profiles for changes in expressed proteins during the growth phase of fiber (trichome) initiation on ovules of a wild-type fiber producing line and a fiberless line [24]. Two of the five uniquely up-regulated proteins in wild-type ovules after fiber initiation were identified as BR isoforms. This is the first report of BR isoforms in cotton ovules and their characterization using heterologous expression in *Pichia pastoris*.

## 2. Materials and methods

### 2.1. Protein extraction and sequencing

Two proteins designated BR1 and BR2 and reported by Turley and Ferguson [24] to be up-regulated in ovules after fiber initiation were targeted for protein sequencing. These proteins were purified by 2D-PAGE with modifications to the Turley and Ferguson protocol [24]. These modifications included electrophoresis of 500 μg of protein from 4 DPA ovules of DP 5690 in four IEF tube gels (2.0 mg total) with dimensions of 4 mm i.d. and 140 mm long. Focusing and second gels were performed by standard methods and staining was performed using Coomassie blue R-250, 50% ethanol and 10% acetic acid overnight and destained for 1 h in 50% ethanol and 10% acetic acid [24]. Additional destaining occurred in 7% acetic acid and 5% ethanol. The proteins D12 (BR1) and D13 (BR2) were excised and collected in individual tubes (four pieces per ependorf tube). These four pieces of BR1 and BR2 gels were loaded into separate wells of a 15% SDS-PAGE with electrophoresis at 50 mV overnight. SDS-PAGE gels were stained with Coomassie, and protein was excised and placed in a 1.5 mL ependorf tube. These protein bands were sequenced at the Protein Structure Laboratory (University of California at Davis) using in gel trypsin digestion, HPLC purification, followed by the sequencing of two of the HPLC peaks (Edman degredation) with an ABI470 sequencer. The short amino acid sequences were blasted using the tblastn program with the EST_others database with the "limit to entrez queries" set to *Gossypium* (http://ncbi.nlm.nih.gov/BLAST/). Numerous ESTs were identified and full length sequences compiled for both the BR1 and BR2 forms of benzoquinone reductase.

### 2.2. Cloning of cotton 1,4-benzoquinone reductase

Two full length clones encoding isoforms of benzoquinone reductase were amplified from cDNA isolated from 3 DPA fiber of Stoneville 4793R [22] using a single set of primers. Two PCR primers (Integrated DNA Technologies, Skokie, IL)

were designed for sense 5′-GGAGCGAGG<u>GAATTC</u>ATGGCA ACCAAAGTGTATATTG-3′ and the anti-sense 5′-ACGA-CAGGG<u>GGTACC</u>TCAGGCAGCTGTCTTGAGTTTC-3′ amplification using PCR with 92 °C/3 min, 35 cycles of 92 °C/ 30 s and 53 °C/1 min, then 70 °C/30 min. The PCR was performed using the DyNAzyme EXT DNA Polymerase kit (New England Biolabs, Beverly, MA) and generated two 640 bp products with unique EcoRI (5′) and (3′) KpnI restriction sites (underlined) for directional insertion and ligation in frame with the α-factor secretion sequence in the expression vector pPICZαA (Invitrogen, Carlsbad, CA). The PCR product was TA cloned into a TOPO TA pCR®2.1 TOPO vector (Invitrogen, Carlsbad, CA) and positive transformants selected with blue/white screening. Plasmid DNA was isolated using the standard alkali lysis and precipitation by polyethylene glycol protocols [19]. Restriction enzyme digestion of isolated plasmids with HindIII and electrophoresis was used to distinguish between the BR1 (cleaves at 551 bp) and BR2 (cleaves at 269 bp and 551 bp) isoforms. The BR1 and BR2 cDNA clones were sequenced in both directions, and the protein sequence was deduced and analyzed using ExPASy Proteomic tools Translate, ProtParam, MW, p*I*, and Titration curve (http://us. expasy.org/tools/). Both clones were evaluated for rare codon usage patterns in *Pichia pastoris* (http://molbiol.ru/eng/scripts/ 01_11.html).

The full length BR1 (GenBank accession EF217318.1) and BR2 (GenBank accession EF217319.1) cDNA were excised from the TOPO plasmid by EcoRI/KpnI restriction digestion (pPICZαA was processed at the same time), purified by electrophoresis on 1% agarose gels, gel extracted using the QIAquick Gel Extraction kit (Qiagen, Valencia, CA), and ligated in frame with the α-factor secretion sequence in the expression vector pPICZαA. The recombinant plasmids were transformed into TOP10 electrocomp $^{Tm}$cells (Invitrogen, Carlsbad, CA) and screened for inserts in selected colonies using PCR. Recombinant plasmids were designated pGhBR1 and pGhBR2 containing the BR1 and BR2 isoforms, respectively. Plasmids pGhBR1 and pGhBR2 were purified by the standard alkali lysis and precipitation by polyethylene glycol protocols as described by Sambrook et al. [19], linearized with PmeI, then transformed into *P. pastoris* X-33 cells by electroporation using a Gene Pulser (BioRad, Hercules, CA). *P. pastoris* X-33 cells were prepared for transformation by the method of Lin-Cereghino et al. [10]. *P. pastoris* was grown on YPD zeocin plates containing 2% peptone, 1% yeast extract, 2% glucose, 1.5% agar and 100 μg zeocin/mL in an incubator set for 30 °C.

### 2.3. Expression of cotton 1,4-benzoquinone reductase protein in Pichia pastoris

Six colonies were tested for the heterologous expression of BR1 and BR2 in *P. pastoris*. Each colony was suspended in 50 mL of BMG media (100 mM potassium phosphate [pH 6.0], 1.34% YNB, $4 \times 10^{-5\%}$ biotin, and 1% glycerol) and grown overnight in a shaking incubator (240 rpm) at 30 °C in sterile 250 mL baffled flasks. When $OD_{600}$ reached 2.5— 3.5, the suspensions were poured into sterile Oak Ridge tubes

and centrifuged at $3000 \times g$ for 5 min in a SA-600 rotor (Sorvall) at 4 °C. The supernatants were decanted, and the pellets were resuspended in BMM media (100 mM potassium phosphate pH 6.0, 1.34% YNB, $4 \times 10^{-5\%}$ biotin and 1% methanol) and grown for 96 h in a shaking incubator (240 rpm) at 30 °C in sterile 250 mL baffled flasks. Fifteen mL of each sample was collected in 30 mL corex tubes and centrifuged at $5000 \times g$ for 10 min in a SA-600 rotor (Sorvall) at 4 °C. The supernatants were poured into Centriprep YM-10 tubes and concentrated overnight in a SA-600 rotor (Sorvall) at $2500 \times g$ at 4 °C. After the samples were concentrated to 0.7−1 mL volumes, they were placed in 1.5 mL microfuge tubes (Bio-Rad Lab., Hercules, CA) and aliquots were removed and evaluated with SDS-PAGE. Single colonies of BR1 and BR2 with the highest level of excretion were replated on YPD zeocin plates and grown for 2 days.

Once the highest expressing BR1 and BR2 colonies were determined, the process was scaled up using a liter of BMG, inoculated with the transformed *P. pastoris*, then equally divided into three, two liter baffled flask and grown overnight at 30 °C. Four sterile 250 mL Nalgene centrifugation bottles (Nalge Co., Rochester, NY) were filled with the yeast solution, and the yeast were pelleted at $3000 \times g$ for 10 min at 4 °C in a GSA-9847 rotor. The four pellets were resuspended in 1 L of BMM, then equally divided between 3, 2-l baffled flask and grown for 96 h at 30 °C. Four 250 mL Nalgene centrifugation bottles (Nalge Co., Rochester, NY) were filled with the media, and the yeast was pelleted at $3000 \times g$ for 10 min at 4 °C in a GSA-9847 rotor. The supernatant was sterilized by passing through a 1000 mL Filter System (Corning, Corning, NY) and then concentrated in Centriprep YM-10 tubes in a SA-600 rotor (Sorvall) at $2500 \times g$ at 4 °C. Protein concentrations were measured by the Bradford method (Bio-Rad Lab., Hercules, CA) with gamma-globulin as the standard. SDS-PAGE was performed as previously described [24] in 16% gels. The BR enzymes were assayed as reported in *P. chrysoporium* according to Brock et al. [2].

## 3. Results and discussion

### 3.1. 2D-PAGE of developing cotton ovules and protein sequencing

Proteins profiles were evaluated to determine differences between total proteins extracted from ovules before and after fiber initiation, i.e., 3 days before anthesis (DBA) to 4 days post-anthesis (DPA) [24]. A group of 37 proteins with different expression patterns were reported when comparing the protein profiles from the fiberless SL 1-7-1 and the wild-type DP 5690 line. Two of these proteins, BR1 and BR2, originally designated D12 and D13, respectively [24], were very similar in molecular mass (Fig. 1). BR1 and BR2 also shared similar expression patterns developing exclusively in the wild-type line after fiber initiation by 2 and 4 DPA. For protein sequencing, 2D-PAGE was scaled up by loading 0.5 mg phenol extracted protein on four gels to isolate larger quantities of BR1 and BR2 proteins. The amino acid sequences for protein fragments
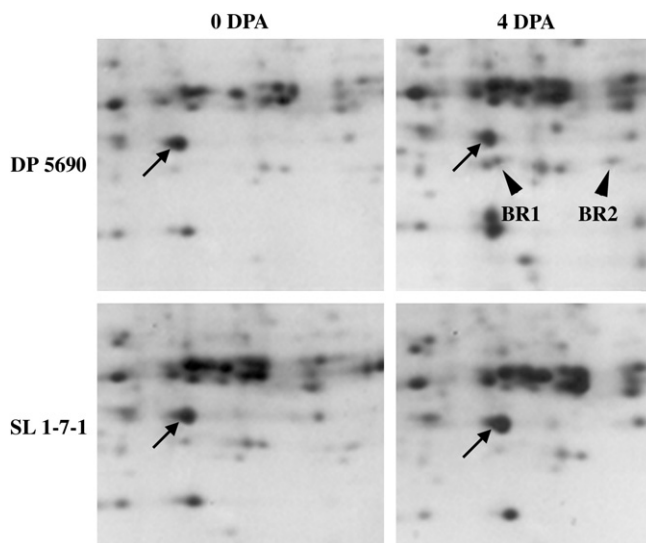


Fig. 1. 2D-PAGE of ovule proteins at 0 and 4 DPA from a wild-type fiber producing cotton (DP 5690) and a fiberless cotton (SL 1-7-1). Stick arrows pinpoint a common protein in all four gels for easy comparison. The solid arrowheads pinpoint the up-regulated BR1 and BR2 in the 4 DPA/DP5690 panel.

of BR1 (one fragment) and BR2 (two fragments) were determined (Table 1). These amino acid sequences were compared using tblastn with the translated NCBI est_others database with the "limits to entrez queries" set to *Gossypium*. Four cotton ESTs were selected (Table 1) to determine a consensus full length sequence, and PCR primers were designed to amplify the coding region of BR1 and BR2.

### 3.2. Cloning and comparison of benzoquinone reductase isoforms

One set of PCR primers was designed to amplify the coding regions of both BR clones. The nucleotide sequences encoding the full length coding region for both BR1 and BR2 were determined to be 609 bp in length and encoded proteins with deduced amino acid sequences of 203 residues (Fig. 2). Fourteen of the 203 residues differ between the amino acid sequences of BR1 and BR2 and are marked in Fig. 2 in bold and underlined. At the carboxyl end of BR2, residue number 201 may have been modified from an A to a T by the 3′ primer used in the PCR reaction. Cotton ESTs for this isoform encode an A at position 201. The theoretical molecular mass and p*I*s were calculated using ExPASy program ProtParam (Table 1) and compared to the reported molecular mass and p*I*s of BR1 and BR2 from 2D-PAGE (Turley and Ferguson, 1996). The calculated molecular mass was approximately 4 kDa larger than the theoretical mass for both BR1 and BR2. No attempts were made to determine if this was the result of a post-translational event or if this was an erroneous calculation by the scanning program. Both BR1 and BR2 were predicted by ExPASy program pSORT to have a high probability of localization to the peroxisome due to an internal AKL sequence (residues 35−37).

Table 1
Comparison of measured and deduced protein parameters of the BR1 and BR2 proteins in cotton

| Feature | Protein | |
|---|---|---|
| | BR1 | BR2 |
| Protein sequence 1 comparison | QVPETLS**E**EILGK | L − QVSETLSEEV |
| Sequenced/deduced seq | QVPETLS**D**EILGK | **LW**QVSETLSEEV |
| Protein sequence 2 comparison | Not determined | A**P**LDATGGL − RTQ |
| Sequenced/deduced seq | | A**F**LDATGGLWRTQ |
| Consensus seq. derived from EST's (accession numbers) | BF274514.2, BF273227.1, BQ405053.1 & BQ405912.1 | BQ406504.1, BQ403554.1, BM360034.1 & BE055387.2 |
| Cotton species | Arboreum & hirsutum | Arboreum & hirsutum |
| Determined kDa/deduced kDa | 25.9/21.8 | 25.7/21.6 |
| Determined p*I*/deduced p*I* | 5.80/6.09 | 5.98/6.20 |
| GenBank accession No. | EF217318.1 | EF217319.1 |
| Protein accession No. | ABN12320.1 | ABN12321.1 |

Standard 1 letter abbreviation for amino acids with amino acids in bold print reflecting differences and "−" representing undetermined amino acid.

**BR1**

```
ATGGCAACCAAAGTGTATATTGTATACTATTCCATGTATGGACATGTGGAAAAACTAGCA
 M  A  T  K  V  Y  I  V  Y  Y  S  M  Y  G  H  V  E  K  L  A

GAGGAGATAAAGAAAGGGGCGGCATCTGTTGAAGGTGTTGAAGCGAAACTATGGCAGGTC
 E  E  I  K  K  G  A  A  S  V  E  G  V  E  A  K  L  W  Q  V

CCTGAGACTCTGTCAGATGAGATTCTTGGAAAGATGAGTGCACCACCAAAAGTGATGTA
 P  E  T  L  S  D  E  I  L  G  K  M  S  A  P  P  K  S  D  V

CCGATCATTACACCACATGACCTTCCTGAGGCTGATGGGTTTATTTTTGGCTTCCCAACA
 P  I  I  T  P  H  D  L  P  E  A  D  G  F  I  F  G  F  P  T

AGATTTGGCATGATGTCTGCCCAATTCAAAGCATTTATGGATGCAACTGGTGGTCTATGG
 R  F  G  M  M  S  A  Q  F  K  A  F  M  D  A  T  G  G  L  W

AGAACTCAGCAACTTGCTGGAAAACCTGCTGGGATATTCTATAGCACTGGATCTCAAGGT
 R  T  Q  Q  L  A  G  K  P  A  G  I  F  Y  S  T  G  S  Q  G

GGCGGACAAGAAACTACACCGTTGACTGCAATCACTCAGCTTGTTCACCACGGGATGATT
 G  G  Q  E  T  T  P  L  T  A  I  T  Q  L  V  H  H  G  M  I

TTTGTTCCCATCGGATACAGCTTTGGTGCTGGCATGTTTGAGATGGAGCAAGTGAAGGGT
 F  V  P  I  G  Y  S  F  G  A  G  M  F  E  M  E  Q  V  K  G

GGAAGCCCCTTACGGTGCAGGAACCTATGCTGGGGACGGAACAAGAATGCCATCCGAACTT
 G  S  P  Y  G  A  G  T  Y  A  G  D  G  T  R  M  P  S  E  L

GAGTTCGCGCAAGCTTTCCACCAAGGGAAGTACATTGCCGGAATCACAAAGAAACTCAAG
 E  F  A  Q  A  F  H  Q  G  K  Y  I  A  G  I  T  K  K  L  K

ACAGCTGCCTGA
 T  A  A  -
```

**BR2**

```
ATGGCAACCAAAGTGTATATTGTATACTATTCCATGTACGGACACGTAGAAAAACTTGCA
 M  A  T  K  V  Y  I  V  Y  Y  S  M  Y  G  H  V  E  K  L  A

GAGGAAATAAGGAAAGGGGCTGCATCTGTTGAAGGTGTTGAAGCCAAATTATGGCAGGTC
 E  E  I  R  K  G  A  A  S  V  E  G  V  E  A  K  L  W  Q  V

TCTGAGACTCTATCGGAAGAAGTTCTTGGAAAGATGAGTGCACCTCCAAAAAGTGATGTA
 S  E  T  L  S  E  E  V  L  G  K  M  S  A  P  P  K  S  D  V

CCAGTTATTACACCGACGATCTTCCTGAGGCCCGATGGGTTTGTTTTTGGTTTCCCAACA
 P  V  I  T  P  N  D  L  A  E  A  D  G  F  V  F  G  F  P  T

AGATTCGGAATGATGTCCGCACAATTCAAAGCTTTTCTGGATGCAACCGGAGGTCTATGG
 R  F  G  M  M  S  A  Q  F  K  A  F  L  D  A  T  G  G  L  W

AGAACTCAGCAACTTGCAGGAAAACCTGCTGGGATCTTCTATAGCACCGGATCACAAGGC
 R  T  Q  Q  L  A  G  K  P  A  G  I  F  Y  S  T  G  S  Q  G

GGTGGACAAGAGACTACAGCGTTGACTGCAATCACTCAACTCGTTCACCATGGGATGATA
 G  G  Q  E  T  T  A  L  T  A  I  T  Q  L  V  H  H  G  M  I

TTTGTTCCCATCGGATACACCTTCGGTGCTGGCATGTTTGAGATGGAGCAAGTGAAAGGT
 F  V  P  I  G  Y  T  F  G  A  G  M  F  E  M  E  Q  V  K  G

GGAAGTCCATATGGTGCGGAACATATGCCGGGGACGGATCAAGAATGCCGTCGGAACTT
 G  S  P  Y  G  A  G  T  Y  A  G  D  G  S  R  M  P  S  E  L

GAGTTGGCACAAGCTTTCCACCAAGGTAAGTACATAGCTGGCATCACAAAGAAACTCAAG
 E  L  A  Q  A  F  H  Q  G  K  Y  I  A  G  I  T  K  K  L  K

ACAGCTGCCTGA
 T  A  A  -
```

Fig. 2. Nucleotide and deduced amino acid sequences of BR1 and BR2 from cotton fiber. The underlined bold residues indicated differences between the amino acid sequences of BR1 and BR2. The underlined nucleotide sequences indicate HindIII sites in BR1 (one site) and BR2 (two sites).

### 3.3. Heterologous expression of 1,4-benzoquinone reductase isoforms in Pichia pastoris

To study the expression of recombinant BR1 and BR2, these clones were independently subcloned into the yeast expression vector pPICZαA in frame with the α-factor secretion sequence and transformed into *Pichia pastoris*. The vector contains a methanol-inducible promoter that allows the expression of the protein and secretion into the yeast media. Six transformed colonies were grown in 250 mL flasks and the secretion of BR1 and BR2 was evaluated using SDS-PAGE and Coomassie staining. The colonies which produced the highest expression of each BR protein were used in subsequent scaling up the production of these proteins. These colonies were grown for 96 h, and the supernatant with the proteins were concentrated.

The BR1 and BR2 proteins were determined to be approximately 26.5 kDa in SDS-PAGE and were the most predominant protein in the media (Fig. 3, lanes B and C). The other four bands were likely yeast proteins as we have seen similar bands in the expression of other heterologous proteins in *P. pastoris*. A control
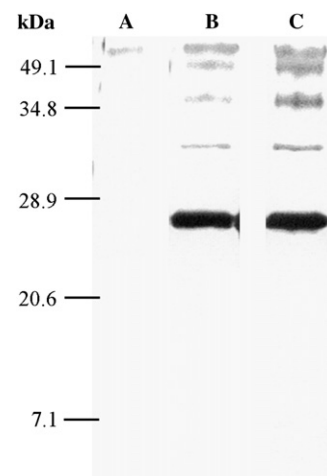


Fig. 3. The heterologous expression of BR1 and BR2 in *P. pastoris*. Lane A is a control lane of pPICZαA without an inserted cDNA clone. Lane B & C are the expressed BR1 and BR2 proteins at an estimated 26.5 kDa.

*P. pastoris* created by transformation with a pPICZαA plasmid without an insert and grown at the same time as BR1 and BR2 under an identical situation did not produce the 26.5 kDa protein (Fig. 3, lane A). Both BR1 and BR2 were measured for activity and found to reduce DMBQ as did the fungal enzymes; however, the specific activity was low, and may reflect a partial inactivation of the protein. The heterologous protein Both BR1 and BR2 are likely cytosolic/peroxisomal enzymes and therefore not processed through the ER. In yeast, BR1 and BR2 pass through the ER and are excreted into the media. The increased size of *P. pastoris* produced BR1 and BR2 compared to the theoretical size as mentioned above (Table 1), may indicate a post-translational modification (glycosylation) which could lead to the partial inactivation of the BR proteins. Further work will be needed to assess this possibility.

Questions arise as to the possible role that cotton BRs may play in developing ovules. Ovules are far removed from the white and brown rot fungi, and parasitic plants. Two clones which are very similar to the cotton BR1 and BR2 may offer some clues. These clones are: first, the FQR1 flavodoxin-like quinone reductase (NP200261; 5e−90) which is a primary auxin response gene in *Arabidopsis* and has a 10-fold induction of its mRNA after 30 min of indole-3-acetic acid treatment [9] and second, the quinone oxidoreductase QR2 (AAG53945; 2e−83) which is up-regulated in roots as a primary response to allelopathic quinones [12].

The first clone, FQR1 flavodoxin − like quinone reductase, is interesting because it is a auxin response gene. Auxin is one of the plant growth regulators which induces fiber development and has been reported to increase in wild-type ovules before fiber initiation [18]. We have not localized the BR to any tissue of the ovule; however, an examination of ESTs encoding BR in the NCBI database indicates that the most similar transcripts (*E* values of 0.0) are derived from six different cDNA libraries constructed from mRNA isolated from 5−10 and 20 DPA fiber from *G. hirsutum* lines Xu-142, *Acala maxxa*, DES 119 and DP 90 [7,20,23,25] and 7−10 DPA fiber of *G. arboreum* [25], and from three libraries for developing stems [23]. The report of BR transcripts in 20 DPA is confirmed by the presence of BR1 (protein 22) in 21 DPA fiber [5].

The real problem with this theory comes in the study of the SL 1-7-1 ovules. No one has yet evaluated the auxin levels in the SL 1-7-1 line; however, most likely they are similar to the wild-type line. The fact that most of the reported BR ESTs are from developing cotton fiber may indicate the BR genes are enriched in the fiber/epidermis/seed coat. This could make the visualization of BR more of a threshold issue where the protein is present but in reduced quantities in SL 1-7-1 fiberless ovules (fiber constitutes a large amount of tissue at 2 and 4 DPA). As monospecific polyclonal antibodies are developed, this issue can be resolved.

The second clone is the quinone oxidoreductase QR2 which is up-regulated in roots as a primary response to allelopathic quinones [12,26]. We have reported in this paper that the heterologous cotton BR has similar activities to DMBQ as BR homologues expressed in the rot fungi *P. chrysosporium* and *G. trabeum* [1,2,15]. Cotton ovules do not seem to have a large external source of quinones; however, directly before fiber initiation, numerous cytological changes are observed in the seed coat [16]. In 1 DBA ovules it was observed that numerous small vacuoles were filled with an electron dense material putatively identified as a phenolic-type compound [16]. This electron-dense material from the vacuoles began to disappear from the vacuoles around 16 h before anthesis that consistently coincided with fiber initiation. This could be a source of metabolites for BR in cotton ovules. Other sources may be derived from the peroxidases which are involved in cross linking pectic polysaccharides with phenolic acids in cell walls [6,13,14] or the apparent natural occurrence of DMBQ in cell walls [8].

Presently there are many possibilities for the induction of cotton BR's during fiber initiation. Another possibility is the induction during plant stress. Rice BR has recently been reported to be up-regulated in line IR651 when grown under saline conditions [11]. Future efforts will attempt to identify the possible roles of BR in fiber development and possible environmental/cellular signals for induction.

## 4. Conclusion

Two isoforms of BR were determined to be up-regulated during early fiber initiation in cotton ovules which produce fiber. These isoforms have been sequenced, cloned and expressed in a *Pichia pastoris* expression system. The heterologous proteins are similar in both molecular mass and p*I* to the BR proteins found in 2D-PAGE. The one exception is in activity, the heterologous protein does reduce DMBQ but has a low specific activity. The function of BR in the fiber is not yet known, but numerous possibilities exist which need to be evaluated. Is BR upregulated by an increase in auxin or the presence of allelopathic quinones (both reported before fiber initiation)? The presence of BR mRNA in older fiber would suggest that this protein has a function in both the elongating and secondary cell wall. Further work is needed to determine the importance of the BR isoforms in fiber development.

## Acknowledgements

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